

## TREATMENT OF INFLAMMATION

### FIELD OF THE INVENTION

This invention relates to the field of therapeutics and in particular to the treatment of inflammatory conditions.

### PRIOR ART

The following is a list of art which is considered to be pertinent for describing the state of the art in the field of the invention. Acknowledgement of these references herein will at times be made by indicating their number within brackets from the list below.

1. Fishman P, et al. Evidence for involvement of Wnt signaling pathway in IB-MECA mediated suppression of melanoma cells. *Oncogene*, 21:4060-4064 (2002).
2. Fishman P, et al. Targeting the A<sub>3</sub> adenosine receptor for cancer therapy: inhibition of Prostate carcinoma cell growth by A<sub>3</sub>AR agonist. *Anticancer Res*, 23:2077-2083 (2003).
3. Madi L, et al. A<sub>3</sub> adenosine receptor activation in melanoma cells: association between receptor fate and tumor growth inhibition. *J. Bio. Chem.*, 278:42121-42130 (2003).
4. Ohana G, et al. Inhibition of primary colon carcinoma growth and liver metastasis by the A<sub>3</sub> adenosine receptor agonist IB-MECA. *British J. Cancer.*, 89:1552-1558 (2003).

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5. Fishman P, et al. An agonist to the A<sub>3</sub> adenosine receptor inhibits colon carcinoma growth in mice via modulation of GSK-3 $\beta$  and NF- $\kappa$ B. *Oncogene*, **23**:2465-2471 (2004).
6. US Patent Application publication No. 2004016709A1.
7. Szabo, C., et al. Suppression of macrophage inflammatory protein (MIP)-1 $\alpha$  production and collagen-induced arthritis by adenosine receptor agonists. *British J. Pharmacology*, **125**:379-387 (1998).
8. Mabley, J., et al. The adenosine A<sub>3</sub> receptor agonist, N<sup>6</sup>-(3-iodobenzyl)-adenosine -5'-N-methyluronamide, is protective in two murine models of colitis. *Europ. J. Pharmacology*, **466**:323-329 (2003).
9. Baharav, E., et al. The effect of adenosine and the A<sub>3</sub> adenosine receptor agonist IB-MECA on joint inflammation and autoimmune diseases models. *Inter. J. Mol. Med.* **10**(supplement 1) page S104, abstract 499 (2002).
10. Bahave E. et al. Antiinflammatory effect of A<sub>3</sub> Adenosine receptor agonists in murine autoimmune arthritis models. *J. Rheumatolog* **32**:469-476 (2005);
11. Montesinos, M. Carmen, et al. Adenosine A<sub>2A</sub> or A<sub>3</sub> receptors are required for inhibition of inflammation by methotrexate and its analog MX-68. *Arthritis & Rheumatism*, **48**:240-247 (2003).
12. Madi L, et al. The A<sub>3</sub> Adenosine Receptor is Highly Expressed in Tumor vs. Normal Cells: Potential Target for Tumor Growth Inhibition. *Clinical Cancer Research*, **10**: 4472-4479, (2004).
13. Gessi, S. et al. Elevated expression of A<sub>3</sub> adenosine receptors in human colorectal cancer is reflected in peripheral blood cells *Clinical Cancer Research* **10**:5895-5901, (2004).
14. US Patent Application No. 20040137477 A1.

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15. Chan ES and Cronstein BN."Molecular action of methotrexate in inflammatory diseases".*Arthritis research*. 4:266, (2002).
16. Montesinos MC; et al. Adenosine A2A or A3 receptors are required for inhibition of inflammation by methotrexate and its analog MX-68".*Arthritis & rheumatism*.48:240 (2003).
17. Yednock, TA. et al. Methods and compositions for treating rheumatoid arthritis, US Patent Application Publication No.20050074451.
18. Yednock, TA. et al. Methods and compositions for treating rheumatoid arthritis. US Patent Application Publication No.20050065192.
19. Feldman M. et al. Anti-TNF Antibodies and Methotrexate in the Treatment of Autoimmune Disease. AU200051825.
20. Alison MB et al. Combination therapy using a TNF binding protein for treating TNF-mediated diseases. US Patent Application Publication No. US2002119924.

## BACKGROUND OF THE INVENTION

### **A<sub>3</sub> adenosine receptor**

The A<sub>3</sub> adenosine receptor, a G<sub>i</sub> protein-associated cell surface receptor, has been proposed as a target to combat cancer and inflammation. The receptor is highly expressed in various tumor cell types while low expression was shown in adjacent normal tissues. Activation of the receptor by a specific synthetic agonist induces de-regulation of signal transduction pathways which include the Wnt and the NF- $\kappa$ B, resulting in tumor growth inhibition (1-5). *In vivo* studies have shown that A<sub>3</sub>AR agonists inhibit the development of colon, prostate and pancreatic carcinomas as well as melanoma and hepatoma.

A<sub>3</sub>AR agonists were also been shown to act as anti-inflammatory agents by ameliorating the inflammatory process in different experimental autoimmune

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models such as rheumatoid arthritis, Multiple sclerosis and Crohn's disease (6-10). It was proposed earlier that the A<sub>2A</sub> and A<sub>3</sub> receptors mediate the anti-inflammatory effects of methotrexate (11).

A<sub>3</sub> adenosine receptor (A<sub>3</sub>AR) expression levels are elevated in cancer cells as compared to normal cells (12, 13). Thus, the A<sub>3</sub>AR expression level has been described as a mean for the diagnosis of cancer (14). In addition, A<sub>3</sub>AR expression level has also been described to be elevated in peripheral blood mono-nuclear cells of patients with colorectal cancer (13).

### **Methotrexate**

Methotrexate (MTX) is a metabolic antagonist which was initially developed as therapeutic drug for malignant tumors and is currently used, in lower doses, for treating rheumatoid arthritis as well as other autoimmune and allergic diseases.

Although MTX was first introduced as an antiproliferative agent that inhibits the synthesis of purines and pyrimidines for the therapy of malignancies, it is now clear that many of the anti-inflammatory effects of methotrexate are mediated by adenosine (15). In confirmation of this mechanism of action, studies in both animals and patients suggested that adenosine-receptor antagonists, among which is caffeine, reverse or prevent the anti-inflammatory effects of methotrexate (15, 16).

Combination therapies comprising MTX and an additional agent have been suggested: for example, the combination of MTX with an antibody or an inhibitor of  $\alpha$ -4-integrin (17, 18), and the combination of MTX with anti-TNF antibodies or TNF-binding protein in the treatment of TNF-mediated disorders, including autoimmune diseases (19, 20).

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## SUMMARY OF THE INVENTION

The present invention is based on the finding that a combined treatment of the A<sub>3</sub> adenosine receptor (A<sub>3</sub>AR) agonist IB-MECA and MTX led to a greater anti-inflammatory effect in an inflammatory arthritis animal model than treatment with MTX alone or with an A<sub>3</sub>AR agonist alone.

Thus, the present invention provided a method of treating a subject having an inflammatory condition, comprising administering to the subject a combination of an effective amount of MTX and an effective amount of A<sub>3</sub>AR agonist.

Further, the invention provides a method of treating a subject having an inflammatory condition and treated with MTX, comprising administering to the subject an effective amount of an A<sub>3</sub>AR agonist.

Yet further, the invention provides a method of treating a subject having an inflammatory condition and being treated with an A<sub>3</sub>AR agonist, comprising administering to the subject an effective amount of MTX.

The invention also provides the use of an A<sub>3</sub>AR agonist for the preparation of a pharmaceutical composition for treating a subject having an inflammatory condition and being treated with MTX.

The invention further provides the use of MTX for the preparation of a pharmaceutical composition for treating a subject having an inflammatory condition and being treated with an A<sub>3</sub>AR agonist.

The invention still further provides a pharmaceutical composition for treating a patient having an inflammatory condition and being treated with MTX, comprising an effective amount of an A<sub>3</sub>AR agonist.

The invention yet further provides a pharmaceutical composition for treating a patient having an inflammatory condition and being treated with an A<sub>3</sub>AR agonist, comprising an effective amount of MTX.

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The A<sub>3</sub>AR agonists, in accordance with the invention, are preferably, but not exclusively, N<sup>6</sup>- (3-iodobenzyl)-adenosine- 5'-N-methyl-uronamide (IB-MECA) and 2-chloro-N<sup>6</sup>- (3-iodobenzyl)-adenosine- 5'-N-methyl-uronamide (Cl-IB-MECA). However, these currently preferred A<sub>3</sub>AR agonists are by no means exclusive and other such agonists may also be used, as detailed further below.

A preferred, but not exclusive, inflammatory condition to be treated in accordance with the invention is an autoimmune disorder, more preferably Rheumatoid Arthritis (RA). The combination therapy in accordance with the invention may indeed also be applied for other diseases in which either MTX or an A<sub>3</sub>AR agonist is or may be indicated for treatment, as further detailed below.

## **BRIEF DESCRIPTION OF THE DRAWINGS**

In order to understand the invention and to see how it may be carried out in practice, a preferred embodiment will now be described, by way of non-limiting example only, with reference to the accompanying drawings, in which:

**Figs. 1A-1B** are graphical representations of results of an experiment showing the change in severity of arthritis as a function of time in AIA animals treated with either methotrexate (MTX; 0.25 mg/Kg in **Fig. 1A** and 0.25 or 0.75 mg/Kg in **Fig. 1B**), IB-MECA (CF101), or a combination of MTX and IB-MECA or with vehicle only (control).

**Figs. 2A-2B** are bar graphs showing the effect of combined treatment of IB-MECA (CF101) and MTX on the proliferation of human fibroblasts like synoviocytes (FLS) (**Fig. 2A**) or rat FLS (**Fig. 2B**) as determined by MTT assay.

## **DETAILED DESCRIPTION OF THE INVENTION**

The invention is described in the following detailed description with reference to therapeutic methods for the treatment of inflammatory conditions involving administration of a combination of an A<sub>3</sub>AR agonists and MTX. It should

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be noted that in addition to said therapeutic methods, also encompassed within the present invention is the use of each one of the active agents, MTX and an A<sub>3</sub>AR agonist, for the preparation of a pharmaceutical composition for administration to a subject suffering from a inflammatory condition and being indicated for treatment by an anti-inflammatory treatment that comprises use of the other active agent; as well as a pharmaceutical composition for the treatment of inflammatory conditions indicated for treatment by one of these active agents, that comprises an effective amount of the other of these active agents and a pharmaceutically acceptable carrier.

As used in the specification and claims, the forms "*a*", "*an*" and "*the*" include singular as well as plural references unless the context clearly dictates otherwise. For example, the term "*an A<sub>3</sub>AR agonist*" includes one or more agonists.

Further, as used herein, the term "*comprising*" is intended to mean that the methods or composition includes the recited elements, but not excluding others. Similarly, "*consisting essentially of*" is used to define methods and compositions that include the recited elements but exclude other elements that may have an essential significance on the anti-inflammatory activity. For example, a composition consisting essentially of an A<sub>3</sub>AR agonist will not include or include only insignificant amounts (amounts that will have an insignificant effect on the anti-inflammatory effect of the composition) of other active ingredients that have an anti-inflammatory activity. Also, a composition consisting essentially of the active agents as defined herein would not exclude trace contaminants from the isolation and purification method, pharmaceutically acceptable carriers, such as phosphate buffered saline, excipients, preservatives, and the like. "*Consisting of*" shall mean excluding more than trace elements of other elements. Embodiments defined by each of these transition terms are within the scope of this invention.

Further, all numerical values, e.g., concentration or dose or ranges thereof, are approximations which are varied (+) or (-) by up to 20%, at times by up to 10%

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of from the stated values. It is to be understood, even if not always explicitly stated that all numerical designations are preceded by the term "*about*". It also is to be understood, although not always explicitly stated, that the reagents described herein are merely exemplary and that equivalents of such are known in the art.

As detailed in the following exemplary embodiment, the invention is based on the finding that treatment of animals having induced inflammatory arthritis (Adjuvant induced Arthritis, AIA) with IB-MECA, an A<sub>3</sub>AR agonist, in combination with MTX resulted in a combined anti-inflammatory effect, significantly larger than any of these drugs alone.

Thus, in accordance with the invention, there is provided a method for the treatment of an inflammatory condition, the method comprises combined administering to a subject in need of said anti-inflammatory treatment of an effective amount of MTX and an effective amount of A<sub>3</sub>AR agonist.

A combined administration in the context of the present invention denotes administering to a patient the A<sub>3</sub>AR agonist and MTX within the same treatment course; namely during a treatment period, that can last weeks, months or years. Both active agents are given to the patients, each one according to its specific administration schedule. MTX is typically given to patients once weekly, either orally or parentally. An A<sub>3</sub>AR agonist may be administered to a patient orally or parentally, once daily, twice daily, several times daily, every other day, etc. A combined treatment in accordance with the invention may involve, for example, weekly administration of MTX and once or twice daily administration of an A<sub>3</sub>AR agonist. A combination treatment in accordance with the invention is a treatment involving the above combined administration.

A combined treatment according to the invention may be indicated to patients not previously treated by either MTX or an A<sub>3</sub>AR agonist, or to patients treated with either MTX or an A<sub>3</sub>AR agonist that are either not responding properly to the existing treatment or for the purpose of amplifying the therapeutic response.



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Thus, when referring to combined administrations, the invention also pertains to the use of an A<sub>3</sub>AR agonist for the preparation of a pharmaceutical composition for treating a subject having an inflammatory disease and being treated with MTX, or alternatively, to the use of MTX for the preparation of a pharmaceutical composition for treating a subject having an inflammatory disease and being treated with an A<sub>3</sub>AR agonist.

The combined treatment of the two active principles produces an anti-inflammatory effect. Herein, the term "*anti-inflammatory*" will be used to denote the disease modifying effect achieved by the combined treatment in alleviating the inflammatory response in inflammatory conditions. The anti-inflammatory response may be determined on the basis of various parameters as known to the practitioner. For example, when the inflammatory condition is arthritis, the parameters may include one or more of the following: histological parameters, blood parameters of inflammation, the extent of swollen and tender joints, motility parameters, reduction in pain, a number of different overall performance scoring systems, etc.

Thus, the term "*inflammatory condition*" in accordance with the invention shall mean any state of active or sub-clinical inflammatory condition, including immune induced pathologies, that is characterized by a persistent inflammatory response with pathologic sequelae. This state may be characterized by infiltration of mononuclear cells, proliferation of fibroblasts and small blood vessels, increased connective tissue, and tissue destruction.

The term "*inflammatory condition*" may include a variety of conditions associated with inflammatory responses and immune induced pathologies mediated (e.g. autoimmune disorders) by the immune system. However, in accordance with a preferred embodiment, the term "*inflammatory condition*" denotes such conditions in which treatment with MTX is currently indicated. Without being limited thereto, in the context of the present invention, inflammatory conditions include psoriasis,

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psoriatic arthritis, Crohn's disease, rheumatoid arthritis as well as other rheumatic diseases, including polymyositis and systemic lupus erythematosus

As to the active principles, i.e. A<sub>3</sub>AR agonists and MTX, both are known in the art. Generally, the A<sub>3</sub>AR agonist is any compound that is capable of specifically binding to the adenosine A<sub>3</sub> receptor ("A<sub>3</sub>R"), thereby fully or partially activating said receptor thereby yielding a therapeutic effect (in this particular case, an anti-inflammatory effect). The A<sub>3</sub>AR agonist is thus a molecule that exerts its prime effect through the binding and activation of the A<sub>3</sub>AR. This means that at the doses it is being administered it essentially binds to and activates only the A<sub>3</sub>R.: In a preferred embodiment, the A<sub>3</sub>AR agonist has a binding affinity (K<sub>i</sub>) to the human A<sub>3</sub>AR of less than 1000 nM, desirably less than 500 nM, advantageously less 200 nM and even less than 100 nM, typically less than 50 nM, preferably less than 20 nM, more preferably less than 10 nM and ideally less than 5 nM. The lower the K<sub>i</sub>, the lower the dose of the A<sub>3</sub>AR agonist (that may be used) that will be effective in activating the A<sub>3</sub>R and thus achieving a therapeutic effect.

It should be noted that some A<sub>3</sub>AR agonists can also interact with and activate other receptors with lower affinities (namely a higher K<sub>i</sub>). A molecule will be considered an A<sub>3</sub>AR agonists in the context of the invention (namely a molecule that exerts its prime effect through the binding and activation A<sub>3</sub>R) if its affinity to the A<sub>3</sub>R is at least 3 times (i. e. its K<sub>i</sub> to the A<sub>3</sub>R is at least 3 times lower), preferably 10 times, desirably 20 times and most preferably at least 50 times larger than the affinity to any other of the adenosine receptors.

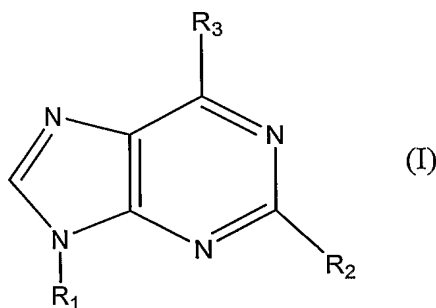
The affinity of A<sub>3</sub>AR agonists to the human A<sub>3</sub>R as well as its relative affinity to the other human adenosine receptors can be determined by a number of assays, such as a binding assay. Examples of binding assays include providing membranes or cells having the receptor and measuring the ability of the A<sub>3</sub>AR agonist to displace a bound radioactive agonist; utilizing cells that display the respective human adenosine receptor and measuring, in a functional assay, the

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ability of the A<sub>3</sub>AR agonist to activate or deactivate, as the case may be, downstream signaling events such as the effect on adenylate cyclase measured through increase or decrease of the cAMP level; etc. Clearly, if the administered level of an A<sub>3</sub>AR agonist is increased such that its blood level reaches a level approaching that of the K<sub>i</sub> of the other adenosine receptors, activation of these receptors may occur following such administration, in addition to activation of the A<sub>3</sub>R. An A<sub>3</sub>AR agonist is thus preferably administered at a dose such that the blood level that will be attained will give rise to essentially only A<sub>3</sub>R activation.

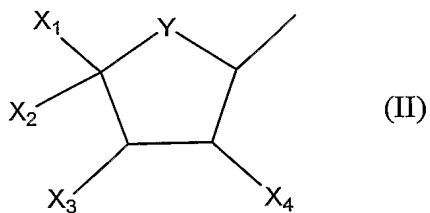
The characteristic of some adenosine A<sub>3</sub>AR agonists and methods of their preparation are described in detail in, *inter alia*, US 5,688,774; US 5,773,423; US 5,573,772; US 5,443,836; US 6,048,865; WO 95/02604; WO 99/20284; WO 99/06053; and WO 97/27173, all of which are incorporated herein by reference.

According to one embodiment of the invention, the A<sub>3</sub>AR agonist is a purine derivative falling within the scope of the general formula (I):



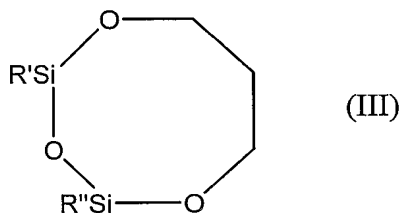
wherein R<sub>1</sub> is C<sub>1</sub>-C<sub>10</sub> alkyl, C<sub>1</sub>-C<sub>10</sub> hydroxyalkyl, C<sub>1</sub>-C<sub>10</sub> carboxyalkyl or C<sub>1</sub>-C<sub>10</sub> cyanoalkyl or a group of the following general formula (II):

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in which:

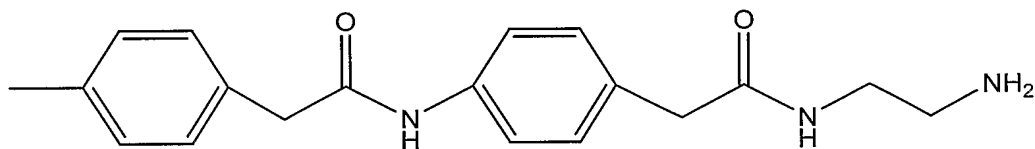
- Y is oxygen, sulfur atom or CH<sub>2</sub>;
- X<sub>1</sub> is hydrogen, C<sub>1</sub>-C<sub>10</sub> alkyl, R<sup>a</sup>R<sup>b</sup>NC(=O)- or HOR<sup>c</sup>-, wherein R<sup>a</sup> and R<sup>b</sup> may be the same or different and are selected from hydrogen, C<sub>1</sub>-C<sub>10</sub> alkyl, amino, C<sub>1</sub>-C<sub>10</sub> haloalkyl, C<sub>1</sub>-C<sub>10</sub> aminoalkyl, C<sub>1</sub>-C<sub>10</sub> BOC-aminoalkyl, and C<sub>3</sub>-C<sub>10</sub> cycloalkyl or are joined together to form a heterocyclic ring containing two to five carbon atoms, and R<sup>c</sup> is selected from C<sub>1</sub>-C<sub>10</sub> alkyl, amino, C<sub>1</sub>-C<sub>10</sub> haloalkyl, C<sub>1</sub>-C<sub>10</sub> aminoalkyl, C<sub>1</sub>-C<sub>10</sub> BOC-aminoalkyl, and C<sub>3</sub>-C<sub>10</sub> cycloalkyl;
- X<sub>2</sub> is hydrogen, hydroxyl, C<sub>1</sub>-C<sub>10</sub> alkylamino, C<sub>1</sub>-C<sub>10</sub> alkylamido or C<sub>1</sub>-C<sub>10</sub> hydroxyalkyl;
- X<sub>3</sub> and X<sub>4</sub> each independently are hydrogen, hydroxyl, amino, amido, azido, halo, alkyl, alkoxy, carboxy, nitrilo, nitro, trifluoro, aryl, alkaryl, thio, thioester, thioether, -OCOPh, -OC(=S)OPh or both X<sub>3</sub> and X<sub>4</sub> are oxygen connected to >C=S to form a 5-membered ring, or X<sub>2</sub> and X<sub>3</sub> form the ring of formula (III):



where R' and R'' are independently C<sub>1</sub>-C<sub>10</sub> alkyl;

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- $R_2$  is selected from hydrogen, halo,  $C_1$ - $C_{10}$  alkylether, amino, hydrazido,  $C_1$ - $C_{10}$  alkylamino,  $C_1$ - $C_{10}$  alkoxy,  $C_1$ - $C_{10}$  thioalkoxy, pyridylthio,  $C_2$ - $C_{10}$  alkenyl;  $C_2$ - $C_{10}$  alkynyl, thio, and  $C_1$ - $C_{10}$  alkylthio; and
- $R_3$  is a  $-NR_4R_5$  group with  $R_4$  being hydrogen or a group selected from alkyl, substituted alkyl or aryl-NH-C(Z)-, with Z being O, S, or  $NR^a$ , and
- when  $R_4$  is hydrogen,  $R_5$  being selected from R- and S-1-phenylethyl, benzyl, phenylethyl or anilide groups, each said groups being unsubstituted or substituted in one or more positions with a substituent selected from  $C_1$ - $C_{10}$  alkyl, amino, halo,  $C_1$ - $C_{10}$  haloalkyl, nitro, hydroxyl, acetoamido,  $C_1$ - $C_{10}$  alkoxy, and sulfonic acid or a salt thereof; or  $R_5$  is benzodioxanemethyl, fururyl, L-propylalanyl- aminobenzyl,  $\beta$ -alanyl-amino- benzyl, T-BOC- $\beta$ -alanylaminobenzyl, phenylamino, carbamoyl, phenoxy or  $C_1$ - $C_{10}$  cycloalkyl; or  $R_5$  is a group of the following formula (IV):

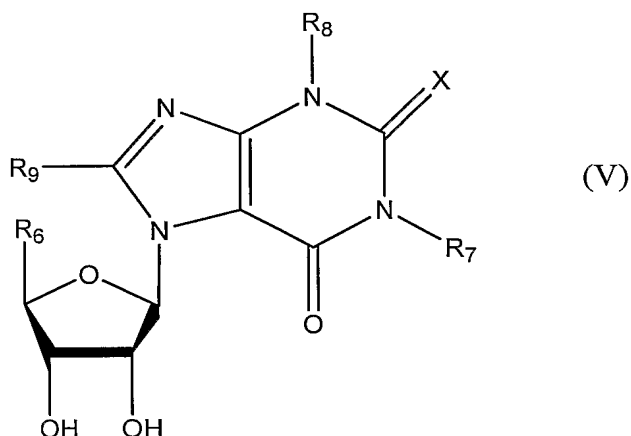


(IV)

or, when  $R_4$  is alkyl, substituted alkyl, or aryl-NH-C(Z)-, then,  $R_5$  is selected from the group consisting of substituted or unsubstituted heteroaryl- $NR^a$ -C(Z)-, heteroaryl-C(Z)-, alkaryl- $NR^a$ -C(Z)-, alkaryl-C(Z)-, aryl- $NR$ -C(Z)- and aryl-C(Z)-;

or the  $A_3AR$  agonist is a xanthine-7-ribose derivative of the following general formula (V):

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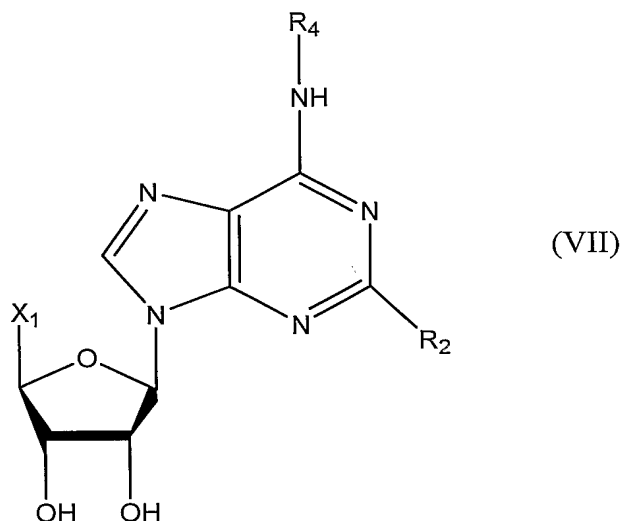
wherein:

- X is O or S;
- $R_6$  is  $R^a R^b NC(=O)-$  or  $HOR^c-$ , wherein
- $R^a$  and  $R^b$  may be the same or different and are selected from hydrogen,  $C_1$ - $C_{10}$  alkyl, amino,  $C_1$ - $C_{10}$  haloalkyl,  $C_1$ - $C_{10}$  aminoalkyl, and  $C_3$ - $C_{10}$  cycloalkyl, or are joined together to form a heterocyclic ring containing two to five carbon atoms; and
- $R^c$  is selected from  $C_1$ - $C_{10}$  alkyl, amino,  $C_1$ - $C_{10}$  haloalkyl,  $C_1$ - $C_{10}$  aminoalkyl,  $C_1$ - $C_{10}$  BOC-aminoalkyl and  $C_3$ - $C_{10}$  cycloalkyl;
- $R_7$  and  $R_8$  may be the same or different and are selected from  $C_1$ - $C_{10}$  alkyl,  $C_1$ - $C_{10}$  cycloalkyl, R- or S-1-phenylethyl, an unsubstituted benzyl or anilide group, and a phenylether of benzyl group substituted in one or more positions with a substituent selected from  $C_1$ - $C_{10}$  alkyl, amino, halo,  $C_1$ - $C_{10}$  haloalkyl, nitro, hydroxyl, acetamido,  $C_1$ - $C_{10}$  alkoxy, and sulfonic acid;
- $R_9$  is selected from the group consisting of halo, benzyl, phenyl,  $C_3$ - $C_{10}$  cycloalkyl, and  $C_1$ - $C_{10}$  alkoxy;

or a suitable salt of the compound defined above. .

According to a more preferred embodiment, the A3AR agonist is a nucleoside derivative of the general formula (VII):

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wherein  $X_1$ ,  $R_2$  and  $R_4$  are as defined.

A specific group of  $A_3AR$  agonists are the  $N^6$ -benzyladenosine-5'-uronamide derivatives. Some preferred  $N^6$ -benzyladenosine-5'-uronamide derivatives are  $N^6$ -2-(4-aminophenyl)ethyladenosine (APNEA),  $N^6$ -(4-amino-3-iodobenzyl) adenosine-5'-(N-methyluronamide) (AB-MECA) and 1-deoxy-1-{6-[(3-iodophenyl) methyl]amino}-9H-purine-9-yl}-N-methyl- $\beta$ -D-ribofuranuronamide (IB-MECA) and 2-chloro- $N^6$ -(3-iodobenzyl)adenosine-5'-N-methyluronamide (Cl-IB-MECA).

According to another embodiment, the  $A_3AR$  agonist is  $N^6$ -benzyladenosine-5'-alkyluronamide- $N^1$ -oxide or  $N^6$ -benzyladenosine-5'-N-dialkyluronamide- $N^1$ oxide.

The MTX and  $A_3AR$  agonist are administered in amounts which are sufficient to achieve an anti-inflammatory effect. The amount of each active agent (MTX or  $A_3AR$  agonist) is at least the amount which provides the desired anti-inflammatory effect when given alone. Nonetheless, the invention use may be contemplated to provide therapeutic combinations that may lower total dosage of

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each active agent than may be required when each individual drug is used alone. A reduction in adverse effects may also be noted.

As will be appreciated, the amount of each of the active agents will depend on the condition to be treated, the intended therapeutic regiment and the desired therapeutic dose. By way of example, were the dose is 1 mg per day and the desired administration regiment is once daily administration, the amount of the active agent in a pharmaceutical composition comprising same will be 1 mg. where it is intended to divide this daily dose in 2 daily administrations, the amount of the active agent in the pharmaceutical composition will be 0.5 mg.

An amount effective to achieve the desired effect is determined by considerations known in the art. An "*anti-inflammatory effective amount*" for purposes herein must be effective to achieve any one of the following anti-inflammatory effect including, for example, amelioration of undesired symptoms associated with inflammation, prevention of the manifestation of such symptoms before they occur, slowing down progression of an inflammatory condition, slowing down the deterioration of symptoms associated with an inflammatory condition, slowing down any irreversible damage caused by a chronic stage of an inflammatory condition, lessening of the severity or curing an inflammatory condition, improving survival rate or providing more rapid recovery form such a condition.

By way of example, when the inflammatory condition is rheumatoid arthritis (RA, which is also a preferred condition to be treated by the present invention), the effective amount may be exhibited by one or more of the following effects: decreased swelling and tenderness of the joints, decreased in pain in the joints, improved motility and flexibility, slowing of the deterioration of the joints and the surrounding tissue, increase in the time period of the remission between acute attacks of the disease, decrease in the time period of the acute attack, prevention of the deterioration of the joints etc.



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It is appreciated that the effective amount depends on a variety of factors including the affinity of the active agent to its corresponding receptor, its distribution profile within the body, a variety of pharmacological parameters such as half life in the body, on undesired side effects, if any, on factors such as age and gender of the subject to be treated, etc. The effective amount is typically tested in clinical studies having the aim of finding the effective dose range, the maximal tolerated dose and the optimal dose. The manner of conducting such clinical studies are well known to a person versed in the art of clinical development.

An amount may also at times be determined based on amounts shown to be effective in animals. It is well known that an amount of X mg/Kg administered to rats can be converted to an equivalent amount in another species (notably humans) by the use of one of possible conversions equations well known in the art. Examples of conversion equations are as follows:

**Conversion I:**

Species	Body Wt. (Kg)	Body Surf. Area (m <sup>2</sup> )	Km Factor
Mouse	0.2	0.0066	3.0
Rat	0.15	0.025	5.9
Human Child	20.0	0.80	25
Adult	70.0	1.60	37

Body Surface area dependent Dose conversion: Rat (150g) to Man (70 Kg) is 1/7 the rat dose. This means that in the present case 0.001-0.4 mg/Kg in rats equals to about 0.14-56 microgram/Kg in humans; assuming an average weight of 70 Kg, this would translate into an absolute dosage of about 0.01 to about 4 mg.

**Conversion II:**

The following conversion factors: Mouse = 3, Rat = 67. Multiply the conversion factor by the animal weight to go from mg/Kg to mg/m<sup>2</sup> for human dose equivalent.

Species	Weight (Kg)	BSA (m <sup>2</sup> )
Human	70.00	1.710
Mouse	0.02	0.007
Rat	0.15	0.025
Dog	8.00	0.448

According to this equation the amounts equivalent to 0.001-0.4 mg/Kg in rats for humans are 0.16-64 µg/Kg ; namely an absolute dose for a human weighing about 70 Kg of about 0.011 to about 4.4 mg, similar to the range indicated in Conversion I.

**Conversion III:**

Another alternative for conversion is by setting the dose to yield the same plasma level or AUC as that achieved following administration to an animal.

Thus, based on measurement made in mice following oral administration of IB-MECA and based on such measurements made in humans in a clinical study in which IB-MECA was given to healthy male volunteers it was concluded that a dose of 1 microgram/Kg – 400 microgram/Kg in mice in which IB-MECA was effective and is equivalent to a human dose of about 0.14 – 57 microgram/Kg, namely a total dose for a 70 Kg individual of 0.01 – 4 mg.

Further, based on the above conversion methods, a preferred dosage range for IB-MECA and Cl-IB-MECA (preferred A<sub>3</sub>AR agonists in accordance with the invention) would be less than 4 mg, typically within the range of about 0.01 to about 2 mg (about 0.14 – 28 micrograms/Kg, respectively) and desirably within the

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range of about 0.1 to 1.5 mg (about 1.4 – 21 micrograms/Kg, respectively). This dose may be administered once, twice or at times several times a day.

Human studies as described in US patent application publication No. 20050101560 and by Fishman et al. [Fishman P. et al., Tolerability, pharmacokinetics, and concentration-dependent hemodynamic effects of oral CF101, an A<sub>3</sub> adenosine receptor agonist, in healthy young men *Int J Clin Pharmacol Ther.* **42**:534-542, 2004] showed that the level of IB-MECA decays in the human plasma with a half life of about 8-10 hours, as compared to a half life of only 1.5 hours in mice, in case of multiple daily administration, correction in the dosages for accumulative effects needs to be made at times (a subsequent dose is administered before the level of a previous one was decayed and thus, there is a build-up of plasma level over that which occurs in a single dose. On the basis of said human trials twice daily administration appears to be a preferred administration regiment. However this does not rule out other administration regiments.

Further, by way of example, an effective amount of MTX is typically in the range of 5 to 25 mg, administered once weekly, orally or parentally.

In accordance with a preferred embodiment, the combined treatment includes weekly administration to a subject of MTX, the dose being in the range between about 5 to 25 mg, concomitant with daily administration, between once and a few times a day, preferably once or twice a day, of A<sub>3</sub>AR agonist, the dose in each administration being in the range of between about 1 to about 1000µg/kg body weight, preferably less than 400µg/kg body weight, and even less than 200µg/kg body weight. Typically, the dose of A<sub>3</sub>AR agonist is in a range of 1 to 100 µg/kg body weight.

A "**composition**" in the context of the invention is intended to mean a combination of the active agent(s), together or separately, with a pharmaceutically acceptable carrier as well as other additives. The carrier may at times have the effect of the improving the delivery or penetration of the active ingredient to the

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target tissue, for improving the stability of the drug, for slowing clearance rates, for imparting slow release properties, for reducing undesired side effects etc. The carrier may also be a substance that stabilizes the formulation (e.g. a preservative), for providing the formulation with an edible flavor, etc. For examples of carriers, stabilizers and adjuvants, see E.W. Martin, REMINGTON'S PHARMACEUTICAL SCIENCES, MacK Pub Co (June, 1990).

MTX and the A<sub>3</sub>AR agonist may be administered to the subject by a variety of delivery modes as known in the art. While MTX may be administered orally or by parenteral injection, it is preferable that the A<sub>3</sub>AR agonist be administered orally. The carrier will be selected based on the desired form of the formulation and the A<sub>3</sub>AR agonist composition may be in the form of a pill, capsule, in the form of a syrup, an aromatic powder, and other various forms.

The invention has been described in an illustrative manner, and it is to be understood that the terminology which has been used, is intended to be in the nature of words of description rather than of limitation. Obviously, many modifications and variations of the present invention are possible in light of the above teaching. It is therefore, to be understood that within the scope of the appended claims, the invention may be practiced otherwise than as specifically described hereinafter.

## **DETAILED DESCRIPTION OF EXEMPLARY EMBODIMENTS**

### **EXAMPLE 1: In vivo Studies**

#### ***Materials***

The A<sub>3</sub>AR agonist, CF101, a GMP grade of the compound known generically as 1-deoxy-1-[6-[[[(3-iodophenyl)methyl]amino]-9H-purine-9-yl]-N-methyl-D-ribofuranuronamide (IB-MECA), was synthesized by Albany Molecular Research Inc. (Albany, NY, USA).

Methotrexate was purchased from Abic, Israel.

### ***Methods***

Female Lewis rats, aged 8-12 weeks were obtained from Harlan Laboratories (Jerusalem, Israel). Rats were maintained on a standardized pelleted diet and supplied with tap water. Experiments were performed in accordance with the guidelines established by the Institutional Animal Care and Use Committee at Can-Fite BioPharma, Petah Tikva, Israel. The rats were injected subcutaneously (SC) at the tail base with 100 µl of suspension composed of incomplete Freund's adjuvant (IFA) with 10 mg/ml heat killed *Mycobacterium tuberculosis*, (Mt) H37Ra, (Difco, Detroit, USA). Each group contained 10 animals.

Treatment with CF101 (10 µg/kg) was initiated on day 14 after vaccination and was orally administered by gavage, twice daily. Another group was treated with Methotrexate (MTX) (0.25, 0.75 mg/kg) intraperitoneally every 3 days, starting on day 14th after vaccination. The control group in each experiment received vehicle only (DMSO in a dilution corresponding to that of the drugs).

Clinical Disease Activity Score was assessed as follows: the animals were inspected every second day for clinical arthritis. The scoring system ranged from 0-4 of each limb: 0- no arthritis; 1- redness or swelling of one toe/finger joint; 2- redness and swelling of more than one toe/finger joints, 3-the ankle and tarsal-metatarsal joints involvement. 4- entire paw redness or swelling. The clinical score was calculated by adding the four individual legs' score. The inflammatory intensity was also determined in accordance with the increase in the rat hind paw's diameter, measured by caliper (Mitotoyo, Tokyo, Japan).

### ***Results***

About 21 days after immunization, most of the vehicle treated animals progressively developed arthritis. CF101 treatment (10 µg/kg, given orally twice daily, starting on day 14th after immunization) and methotrexate (MTX) treatment resulted in a significant decrease in disease severity, very similar for both drugs, as was evaluated by the arthritis clinical score. Disease peaked on days 21-25 and

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maximal effect of CF101 or MTX was seen on these days (**Fig. 1A and Fig. 1B**). A combined treatment with CF101 and MTX resulted in an inhibitory effect which is greater than the additive effect obtained with treatment of CF101 alone or MTX alone (**Fig. 1A and Fig. 1B**).

## **EXAMPLE 2: In Vitro Studies**

### ***Human Fibroblast Like Synoviocytes (FLS) cultures***

Human synovial fluid samples were collected from osteoarthritis (OA) patients undergoing paracentesis. The fluid was centrifuged and the supernatant removed. The cells were resuspended in DMEM containing type I collagenase (4mg/ml), for 2 hours, and shaken vigorously at 37°. The released cells in the supernatant were harvested by centrifugation and were cultured in DMEM containing 10% FBS, 2mM glutamine, 100 U/ml penicillin, 100µg/ml streptomycin, 1% non essential amino acids, 1% sodium pyruvate and 20 nM HEPES buffer in a 37°C, 5% CO<sub>2</sub> incubator. After overnight culture, non-adherent cells were removed. The adherent cells (FLS) were subcultured at a 1 : 2 ratio, and the cells from passages 4 through 10 were used in the experiments.

The effect of CF101 in combination with Methotrexate (MTX) on the proliferation of the FLS was tested utilizing an MTT assay. The cells (5x10<sup>4</sup>/ml cells) were incubated in the presence of MTX (1 µM) in 96-well microtiter plates for 72 hours in the growth medium. At the last 24 hours CF101 (10nM) were added to the cultures.

### ***Rat Fibroblast Like Synoviocytes (FLS) cultures***

Synovia tissue from adjuvant induced arthritis rats was collected. The tissue was minced and subjected for digestion in 4mg/ml type I collagenase and 0.25 w/v trypsin in DMEM. The mixture was shaken vigorously for 4 hours at 37°. The released cells were separated from the supernatant by centrifugation and cultured in DMEM containing 15% FCS, 2mM glutamine, 100 U/ml penicillin, 100µg/ml

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streptomycin in a 37°C, 5% CO<sub>2</sub> incubator. After overnight incubation the nonadherent cells were removed. The adherent cells (FLS) were sub-cultured at a 1 : 2 ratio, and the cells from passages 4 through 10 were used in the experiments.

The effect of CF101 in combination with MTX on the proliferation of the FLS was tested utilizing an MTT assay. The cells (5x10<sup>4</sup>/ml cells) were incubated in the presence of MTX (1 µM) in 96-well microtiter plates for 72 hours in the growth medium. At the last 24 hours CF101 (10nM) were added to the cultures.

### ***Results***

**Figs. 2A and 2B** show, respectively, the effect of MTX alone, CF101 alone or combined treatment with MTX and CF101 on the proliferation of human and rat FLS, as evaluated by the MTT assay. As shown, the inhibitory effect of the combined treatment was greater than the effect achieved by each treatment alone.